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SOME FACTORS IN THE DEFENSE MECHANISM AGAINST
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Early in the experimental studies on *Trypanosoma lewisi* it was discovered that rats having spontaneously recovered from an infection are refractory to reinfection (Kanthack, Durham and Blanford¹). The immunity thus acquired lasts, with an occasional exception, for the life of the rat. The numerous immunological studies with *T. lewisi* have been chiefly centered on determining the factors involved in the recovery of rats from an initial infection (Taliaferro,² Regendanz and Kikuth,³ and others).

Our knowledge of the defense mechanism against reinfection is at present meager and largely based on *in vitro* studies. Only a few observations have been made directly on the parasite during the course of reinfection. Laveran and Mesnil⁴ noted that when recovered or hyperimmunized rats are inoculated intraperitoneally with living *T. lewisi* the parasites seldom appear in the circulating blood. They believed that phagocytosis was the chief mechanism involved in both active and passive immunity. Agglutination of the parasites was observed by Laveran and Mesnil, but they did not consider it an important event in the defense mechanism since the parasites were apparently uninjured by the reaction.

Recent workers have generally failed to observe typical phagocytosis of trypanosomes. Taliaferro⁵ has noted trypanosomes attached to leucocytes under conditions similar to the experiments of Laveran and Mesnil and when inactivated immune rat serum was mixed with washed *T. lewisi* and washed guinea pig leucocytes. It would appear that, at the present time, phagocytosis is not considered to be the important or usual manner of destruction and disposal of *T. lewisi* in either the initial infection or in reinfection.

Taliaferro⁵ injected intravenously large quantities of washed adult trypanosomes into recovered rats—but otherwise normal—and also into recovered rats splenectomized during the course of their initial infection. In the first experiments, 27 rats were inoculated from 6 to 325 days after the end of the

initial infection. There was no apparent correlation between the length of time from recovery to the second inoculation and the duration of the reinfection. No reproduction by the parasites was noted in 8 rats whose reinfections lasted from 2 to 3 days. Dividing forms occurred in 6 rats whose reinfections lasted 2 to 13 days and, for the others, the trypanosomes remained in the blood too short a time to determine reproductive activity. In many of the rats recovery was apparently rapid, since "the parasites were swept from the blood in 5 to 15 minutes." To what extent reproduction developed and whether a numerical increase of trypanosomes occurred is not stated. In the splenectomized series, 10 rats were reinfected from 31 to 353 days after the end of the initial infection. The reinfections lasted from 1 to 40 days and in 2 rats "slight variation" of trypanosomes but no division was noted. It was concluded that, in most cases, the trypanocidal antibody is so strong in recovered rats that it is largely effective before the ablastin (reproduction-inhibiting antibody) has time to play any particular rôle. The nature of the trypanocidal antibody has not been determined, but according to Taliaferro,⁶ it is a lytic antibody which may act as an opsonin *in vivo*. *In vivo* studies, thus far, have failed to indicate the specific character of this antibody.

From a review of the literature it would appear that in most attempts to establish reinfection the experimental animal was injected intraperitoneally with a dilute suspension of infective blood. Since trypanosomes injected into the peritoneal cavity must traverse one or more lymph nodes before they can enter the blood stream, the possibility was early considered that in spontaneous recovery the lymph nodes might become "sensitized" and thus produce an effective barrier against the parasites entering the circulation.

In the present study, experiments designed to gain information on this question clearly demonstrated that trypanosomes pass without hindrance

and as readily through the lymph nodes of a rat spontaneously recovered from previous infection as they do through the nodes of a normal, susceptible rat (Augustine^{7,8}), and that they then appear with equal promptness in the circulation. Further *in vivo* studies, herein reported, revealed some phenomena in the defense mechanism against reinfection. From these studies it is apparent that ablastin, the reproduction-inhibiting antibody believed to play an important rôle in recovery from an initial infection,⁶ appears to be absent or non-operative in reinfection. Rapid recovery from reinfection appears to be due entirely to the action of the trypanocidal antibody, to which the dividing trypanosomes are particularly vulnerable, and by which they are first immobilized or killed and are then phagocytosed by the circulating mononuclear leucocytes, and by which the adult parasites are agglutinated but not killed, and are then mechanically removed from the circulation and destroyed in the liver and probably also in the spleen.

MATERIAL AND METHODS

The present experiments were made with the same strain of trypanosomes (*T. lewisi*) used in our earlier studies.^{7,8} It was originally obtained in 1938 by Dr. E. E. Tyzzer from a wild rat trapped in South Boston. The strain has since been maintained in the laboratory in cultures and in albino rats, originally of Wistar stock, and which are believed to be bartonella-free. The experimental rats were of this same laboratory-bred stock.

The procedures in the present series of experiments are essentially the same as those followed in our earlier studies.^{7,8} The experimental rats were injected with great quantities of trypanosomes. To obtain these, rats, previously inoculated and whose bloods were heavily parasitized, were etherized, the thoracic cavity exposed, and bled to death from the heart into a syringe containing a small amount of heparinized saline. The blood was then diluted several times with normal saline and centrifuged at a medium speed until most of the blood cells were thrown down. The supernatant fluid was then removed and centrifuged a second time for about twenty minutes at high speed. The supernatant of this centrifugation was discarded and the trypanosomes were collected with the leucocytic layer of the sediment. More saline was added to the remaining sediment of the first centrifugation, which sediment always contained an appreciable number of trypanosomes, and after one or more centrifugations and separations the trypanosomes collected were added to those already obtained.

The total number of trypanosomes obtained was estimated by the usual hemocytometer method and the lot was then immediately inoculated into the peritoneal cavity of the experimental rat. The parasites in the experimental rat were studied in fresh samples of blood obtained from the tail or a toe, blood films stained by Wright's method, in fresh teased tissue in saline and in fixed sections of tissue stained with eosin and methylene blue or by Delafield's hematoxylin-eosin method. The degree of reproductive activity was evaluated by determining the percentage of parasites with a divided kinetoplast or nucleus in the stained blood preparations. A total of 200 parasites were carefully examined for evidence of cell division. Cells in the process of dividing into two or more units were counted as a single dividing cell.

RESULTS

The first series of experiments were performed to determine if lymph nodes become effective filters against trypanosomes as a result of spontaneous recovery from previous infection. Four rats (Nos. 201, 202, 203, 204) whose initial infections lasted for about 9, 27, 44 and 69 days were each reinoculated intraperitoneally with more than 330 million adult trypanosomes 27, 37, 14 and 68 days, respectively, after the approximate end of their first infections. A fifth experimental rat (No. 307) whose initial infection lasted approximately 48 days was similarly reinoculated 112 days later with young trypanosomes undergoing rapid multiplication; the infection in the donor had been allowed to run for 5 days. Samples of blood from all 5 rats taken 5, 15 and 30 minutes following the inoculations were negative. The first positive sample was obtained after about 40 minutes from rat 202 and samples from the remaining rats were positive within the first hour. The trypanosomes were active and apparently unharmed during their journey from the peritoneal cavity through the lymphatics into the blood stream. Fluid aspirated within 30 hours from the peritoneal cavity of these rats always contained active and apparently normal trypanosomes free from host cells. None was found after 48 hours. There was no evidence of their destruction in the peritoneal cavity.

A sixth rat, No. 310, whose initial infection lasted about 44 days, was similarly reinoculated with 800 million adult trypanosomes 73 days later. Within an hour trypanosomes appeared in the circulation. The blood stream infection was transient, no parasites were found on the following day. Fifty-six days later the rat was again inoculated with 400

million adult trypanosomes. No parasites were seen in the blood samples taken up to 3 hours after the inoculation was made. The rat was then killed by a blow on the base of the skull, its chest opened and the mediastinal lymph nodes examined. Two lower nodes were enlarged and reddish, the nodes directly cephalad to these appeared normal. One of the lower nodes was removed and teased apart in normal saline. Upon microscopical examination of the material, the donor's blood cells appeared in vibrating clusters containing one or more active parasites. One of the smaller, anterior nodes was then removed and similarly examined. The preparation showed normal lymph node structures with the exception of a few trypanosomes all of which appeared normal and were free from any cell attachment. Saline washings from the peritoneal cavity contained myriads of single, normal-appearing trypanosomes.

Clumping of the donor's blood cells was not observed in our former experiments,^{7,8} where the experimental rat had received but a single inoculation. In the present experiment the experimental animal received on three occasions infective material which always contained an appreciable quantity of the donor's blood cells. The phenomena which followed the third injection suggested the possibility that the successive intraperitoneal injections of the blood cells in the inoculum had caused agglutination in the gland.

The following experiment was made to gain information on this very point.

Two rats, Nos. 311a and 311b, litter mates weighing approximately 110 grams each, were inoculated with *T. lewisi* cultured on NNN medium. The infections were first apparent 6 days later and lasted about 49 days. Five days after the end of the infections each rat was inoculated intraperitoneally with 200 million adult trypanosomes, the inoculum purposely contained an appreciable amount of the donor's washed blood cells. Four hours after inoculation the rats were killed by a blow on the base of the skull and the mediastinal lymph nodes examined.

Two other rats, Nos. 312a and 312b, litter mates of 311a and 311b, were, at the beginning of the experiment, given intraperitoneally 2.5 cc. of washed blood cells from a normal rat. Similar inoculations were made at 10-day intervals, each rat receiving a total of 6 inoculations. On the fourth day after the last injection these rats were inoculated with 200 million trypanosomes, of the same material as was used for their litter mates and approximately 4 hours later the rats were killed in a similar manner and the

mediastinal lymph nodes examined. If, in our earlier experiment (rat 310), agglutination in the node were due to an acquired immunity to the trypanosomes alone, we should expect to observe the phenomenon in the nodes of rats 311a and 311b. If, however, the masses were due to the repeated injections of donor blood cells in the inoculum, we should expect agglutination and retention of parasites in lymph nodes of rats 312a and 312b.

Upon gross examination of the mediastinal lymph nodes, one or both of the paired lower nodes of all four experimental rats were distended and reddish in color from absorption of donor blood cells. The cephalad nodes were normal in appearance. These nodes were removed separately and teased apart in saline. Upon microscopic examination of the preparations none showed the slightest evidence of agglutination of the donor's cells. In every case the trypanosomes moved freely among the blood cells without hindrance. It would appear, therefore, that intranodal agglutination of the donor's cells in the case of rat 310 was a singular phenomenon comparable to the occurrence of isoagglutinins and isolysins rarely observed in laboratory animals (Ingebrigtsen,⁹ Fishbein,¹⁰ and others).

From the results of these first experiments it can be concluded that *T. lewisi*, when inoculated into the peritoneal cavity of rats spontaneously recovered from a previous infection, readily progresses into the lymphatics, passes without hindrance through the lymph nodes and promptly appears uninjured in the blood stream. The filtration accomplished here is, therefore, identical to that which occurs when the parasite is similarly inoculated into normal susceptible rats.⁷

MECHANISM IN RECOVERY FROM REINFECTION

The following experiments are chiefly concerned with the interactions of host and parasite following the entrance of the trypanosomes into the circulation of the rat recovered from one or more previous infections. In the first group the experimental rat was inoculated with adult trypanosomes, *i. e.* the infections in the donor rats were all 15 or more days old. In the second group the analogous inoculations were made with trypanosomes from infections only a few days old which showed many of the parasites undergoing multiplication.

A typical experiment of the first group was as follows:

November 10, 1939. Experiment 4.*

* See Augustine⁸ for a detailed account of the initial injection in the experimental rat.

Initial Infection

11:55 A.M. Experimental rat 4, weight 75 gm., injected intraperitoneally with 250 million adult trypanosomes.

12:15 P.M. Trypanosomes present in drop of blood from tail.

November 12.

10:45 A.M. Multiplication of trypanosomes started. Few normal trypanosomes in fluid aspirated from body cavity.

November 16.

10:00 A.M. 185,000 trypanosomes per c.mm. blood. Rate of reproduction diminished. No trypanosomes from peritoneal cavity.

January 13, 1940.

Approximate end of initial infection.

Reinfection

March 27.

11:45 A.M. Weight 240 gm. Blood negative, 400 million washed adult trypanosomes injected intraperitoneally (second inoculation).

12:45 P.M. Small drop of blood from tail contains 2 trypanosomes.

4:45 P.M. Less than 5,000 trypanosomes per c.mm. blood. Numerous free, normal-appearing trypanosomes in fluid aspirated from peritoneal cavity.

March 28.

10:00 A.M. 15,000 trypanosomes per c.mm. blood, normal, no variation in size or shape, no multiplication. Many normal trypanosomes in fluid from peritoneal cavity.

4:00 P.M. No change from that at 10:00 A.M.

March 29.

9:15 A.M. 20,000 trypanosomes per c.mm. blood. Some variability in size noted; 0.5 per cent dividing cells. A new normal trypanosomes in fluid from peritoneal cavity.

2:15 P.M. No change.

9:30 P.M. No change.

March 30.

9:00 A.M. Numerical count unsatisfactory. Clusters consisting of up to 40 to 50 parasites present in blood samples from tail and toes. The trypanosomes are attached by their posterior tips, their flagella free (figs. 1 and 2). Smaller formations consisting of 2, 3, 4, and 5 individuals similarly attached and single parasites also present. The clusters are free of any attachment to the blood cells. Stained smears show an occasional dividing cell included in the clusters of adult trypanosomes. Blood examinations made hourly up

to 5:00 P.M. show no apparent change. No trypanosomes in washings from peritoneal cavity.

March 31.

1:00 A.M. Blood samples taken from tail and toes contain many agglutinated masses of trypanosomes and a few single parasites. Stained smear shows one dividing cell containing 4 nuclei and 4 kinetoplasts in an agglutinated mass of adult trypanosomes.

2:00 A.M. to 6:00 P.M. Samples of blood taken hourly show no apparent change except that fewer trypanosomes were present in the last samples taken.

April 1.

8:30 A.M. No large agglutinated masses. A few formations of 2 and 3 individuals and a few single trypanosomes (all active) present.

1:00 P.M. No agglutinated parasites seen in several samples of blood. A few single, apparently normal adult forms present.

April 2.

10:00 A.M. No trypanosomes found in many samples of blood. Several drops of blood were collected in 3 cc. normal saline of which 3 one-month-old rats (litter mates) Nos. M, N and O each received 1 cc. intraperitoneally.

11:30 A.M. Rat 4 (the experimental animal) reinoculated intraperitoneally with 380 million washed adult trypanosomes.

1:30 P.M. A few single trypanosomes in sample of blood from tail. Fluid aspirated from peritoneal cavity contains myriads of active, apparently normal trypanosomes.

April 3.

9:30 A.M. No trypanosomes found in several samples of blood taken from the tail and toes. Trypanosomes relatively numerous in fluid aspirated from peritoneal cavity, all normally active. Several drops of blood collected in 3 cc. normal saline of which 3 rats, Nos. P, Q and R (litter mates of Nos. M, N, O) each received 1 cc. intraperitoneally. Fluid aspirated from peritoneal cavity of rat 4 similarly inoculated in rat S, 2 weeks old.

April 4.

9:30 A.M. No trypanosomes found in samples of blood or in saline washings for peritoneal cavity of rat 4.

April 8.

2:00 P.M. Rat 4 negative. Blood samples from rats M, N, O, P, Q and R negative. Blood of rat S swarming with trypanosomes.

April 11.

9:15 A.M. Rat 4 negative. Blood samples from rats M, N, O, P, Q and R negative. Blood of rat S swarming with trypanosomes.

April 15.

10:00 A.M. No change in conditions in all rats from that on April 11.

January 14, 1941.

3:00 P.M. Rat 4. Weight 245 gm. Blood negative for trypanosomes. Reinoculated intraperitoneally (third inoculation) with 800 million washed, adult trypanosomes.

3:45 P.M. Trypanosomes in blood sample from tail.

January 15.

9:00 A.M. 105,000 trypanosomes per c.mm. blood. No dividing cells. Numerous active, normal trypanosomes in fluid aspirated from peritoneal cavity.

January 16.

9:00 A.M. 95,000 trypanosomes per c.mm. blood, 0.5 per cent dividing cells. Very few trypanosomes in fluid obtained from peritoneal cavity.

January 17.

9:00 A.M. 80,000 per c.mm. blood in which small agglomerations noted. No parasites in fluid from peritoneal cavity.

January 18.

9:15 A.M. Blood samples show trypanosomes agglutinated. Single trypanosomes also present.

January 19.

9:30 A.M. Blood samples negative. Rat killed with ether. Autopsy shows no gross pathology; tissues removed and fixed for later study.

The experiments of this group quite clearly demonstrate that when recovered rats are injected intraperitoneally with adult trypanosomes the parasites arrive promptly and uninjured into the circulating blood. Reinfection may last for a few minutes only in some rats while in others it may last for several days. The difference in the duration of reinfection is probably due to differences in titer of the trypanocidal antibody in different animals at the time the transfer is made. Reproduction by the parasites usually started where reinfection lasted for 2 days or longer. Reproduction was never successful. The parasites were agglutinated but not killed in blood stream and then mechanically removed before a numerical increase in the parasite population could be evaluated. It appeared that their destruction might then take place in the liver

and spleen by the action of the phagocytic cells of the reticulo-endothelial system.

A study of the stained sections of liver, spleen and bone of rat 4 revealed no pertinent pathology. We could scarcely expect to find evidence of destruction of the parasites in the tissues since several hours may have passed between the disappearance of the parasites from the blood and the killing of the rat for post mortem study.

Precise information on the fate of the agglutinated masses of trypanosomes was obtained from a study with experimental rat No. 20. This rat was reinoculated about 23 days after the end of its initial infection with more than 900 million washed adult trypanosomes. It was killed with ether on the morning of the third day of reinfection at which time the blood was swarming with large agglutinated masses of trypanosomes. The rat was immediately autopsied and tissues fixed for later study. The spleen was 3.5 cm. long, all other organs appeared normal. Bits of liver, spleen and bone marrow teased apart in saline showed fairly numerous active trypanosomes, most of which were single and without attachment to any host cells. A few agglutinated masses were present in the preparations of liver and spleen. Stained sections of the liver show normal-appearing trypanosomes in the larger blood vessels while in the small vessels remnants of agglutinated parasites with entangled flagella are present (fig. 5). The macrophages show evidence of phagocytic activity but we have not been able to identify any of the inclusions as parts or remnants of trypanosomes. Normal-appearing single trypanosomes and small agglomerations of trypanosomes are occasionally found in vessels of the spleen. No remnants of the parasite have been identified in the spleen but the amount of cytoplasmic inclusions in the phagocytes is marked. No parasites were found in stained sections of the femur or in stained smears of bone marrow.

A study of our stained blood smears shows that the parasites started to multiply in every instance where the second or later infection lasted more than 2 days. Size variation occurred first which was followed by the appearance of individuals with double nuclei or double kinetoplasts and an occasional cell containing 4 nuclei and 4 kinetoplasts. Reproduction was not sufficient to cause numerical increase, as the parasites were agglutinated and removed from the circulation before a numerical increase could be evaluated. Dividing cells are frequently found in the agglutinated masses. No evidence was found, either in the fresh blood preparation or in the stained smears, that the agglutinated mass contained dead parasites. The last trypano-

somes seen in the blood following the disappearance of the agglutinated masses have always been adult forms. They have been few in number, frequently only a single parasite in several fresh preparations and are seldom found in the stained smears. Their life span in the blood is short, seldom longer than an hour after the disappearance of the agglutinated masses. The manner of their disposal is not known.

From the results of these and pertinent parts of seventeen other similar experiments it is apparent that when adult trypanosomes are injected into the peritoneal cavity of rats recovered from one or more previous infections they readily enter and progress through the lymphatic system, pass uninjured through lymph nodes and promptly appear in the blood circulation. They are then agglutinated, but not killed, in the blood stream and are mechanically filtered out and destroyed in the internal organs. When the blood stream infection lasted more than 2 days the trypanosomes invariably started to multiply but the parasites were agglutinated and removed from the circulation before any appreciable increase in numbers occurred. It is also apparent from the results obtained here that the trypanocidal antibody is definitely localized in the blood and has no effect upon trypanosomes remaining in the peritoneal cavity.

In the above experiments the time in which the parasites were demonstrable in the blood varied from a few minutes to 5 days. As a rule, the elapsed time from the end of the initial infection to the second infection showed no correlation with the duration of the second infection. Such variation would appear to be due to difference in the ability of different rats to produce and retain trypanocidal antibody, or in other words, differences in the titer of this antibody at the time of reinoculation.

Since there was a definite attempt by the parasites to multiply, it seemed likely that successful, or at least an appreciable multiplication in such animals might develop from inoculations made with young, dividing trypanosomes which, as we previously demonstrated,⁸ continue to multiply without interruption when transferred to a new, susceptible host. The following experiments are concerned with reinfection from dividing trypanosomes and the defense reactions in the immune host.

A typical experiment of this type follows:

April 16, 1940. Experiment 300.

Experimental rat No. 312, weight 220 gm., inoculated intraperitoneally with washed adult trypanosomes. A normal infection (initial infection) developed which terminated approximately May 23.

January 20, 1941. Weight of rat 215 gm.

10:15 A.M. Inoculated with 400 million trypanosomes collected from a rat with an initial infection 5 days old. 4.0 per cent dividing cells.

11:20 A.M. Many trypanosomes present in samples of blood from tail.

January 21.

9:30 A.M. 80,000 trypanosomes per c.mm. blood, 4.0 per cent dividing cells, size variation marked.

January 22.

9:30 A.M. 85,000 trypanosomes per c.mm. blood, 3.0 per cent dividing cells. Size variation marked.

January 23.

9:00 A.M. 50,000 trypanosomes per c.mm. blood, 1.0 per cent dividing cells. Size variation marked.

January 24.

9:00 A.M. 55,000 trypanosomes per c.mm. blood, 0.5 per cent dividing cells. Size variation not prominent.

January 25.

9:30 A.M. Trypanosomes agglutinated, numerical estimation unsatisfactory. Adult trypanosomes only, 0.05 per cent of which show double nuclei.

January 26.

9:45 A.M. Only a single, adult trypanosome present in several fresh blood samples. A total of 9 adult trypanosomes found on two large stained blood smears.

Blood samples taken January 27, 29 and February 20 negative. The rat was in a weakened condition on February 20 and was found dead the morning of February 23.

Experiments of this type show again that trypanosomes, in reinfection, are ultimately agglutinated in the circulation and are then removed from the blood stream. However, where the second inoculation was made from 6 to 18 months after the end of the initial infection the attempt of the parasites to multiply without affecting any appreciable numerical increase was a constant feature. It was also characteristic, as in the above experiment, for the percentage of dividing cells to decrease in number on successive examinations and reproduction to stop just prior to the event of agglutination, leaving only adult trypanosomes or rarely a dividing cell in the blood.

The study of the stained blood smears from rat 312 at first appeared to offer an acceptable explanation for this lack of an increase in the number of trypanosomes during the early stages of reinfection.

Blood smears made on January 21 show nothing unusual. On January 22 and 23, however, remnants of trypanosomes, free flagella, undulating membranes with the kinetoplast attached, and ruptured trypanosomes discharging their contents were frequently noted. Vestiges indicating that the parasite was undergoing division at the time of its destruction are prominent (figs. 3 and 4). It is not possible, however, to determine whether all of the vestiges are derived from dividing cells since, in many instances, the more fragile cell elements, including the nucleus, are degenerate or lacking. The vestiges are single, free of any blood cells, they lie next to uninjured trypanosomes and normal blood cells and are more or less evenly distributed over the entire smear. The relative daily frequency of these remnants in a count of 400 trypanosomes for the present experiment is: 0.05 per cent January 22, 1.5 per cent January 23, 0.05 January 24, and none on January 25. Thus, their structure and their simultaneous presence and disappearance with dividing parasites support the view that they are derived from cells undergoing, or about to undergo, active division.

The above observations suggested a lytic action on the parasite outside the macrophage. They also suggested that the dividing cell might be particularly vulnerable to this extracellular lytic action of the antibody, but to which the adult forms remained nonsusceptible until the titer of the antibody increased to a point at which they, the adult trypanosomes, became agglutinated, but not killed in the circulation, and then were mechanically removed from the circulation. Further studies, in which the entire course of reinfection in the blood stream was viewed with dark-field illumination showed that, while we were correct in the supposition that the dividing parasites may be affected first by the trypanocidal antibody, the chief mechanism of their destruction was found not to be by extracellular lysis.

In the final group of experiments the trypanosomes were inoculated directly into the heart of the recovered rat. The parasites were thus placed directly in the blood and could be constantly observed throughout the course of the blood stream infection. Fresh, undiluted blood preparations were examined at approximately three-minute intervals with direct and darkfield illumination. Blood smears were also made for later study.

The following experiment is typical of this group:

Rat No. 406 was inoculated intraperitoneally on March 10, 1942, with a few adult *T. lewisi*. The infection which developed ran the typical course and

terminated about 39 days later. On May 5, about the 17th day after the end of the initial infection, trypanosomes were collected from 2 young rats which had been inoculated 4 days earlier with adult parasites. The trypanosomes for these donor rats showed marked size variation with 3 to 4 per cent of the cells undergoing active division. The parasites were finally concentrated in 1 cc. saline of which 0.5 cc. were injected directly into the heart of experimental rat 406, previously etherized. As quickly as possible, a very small drop of blood was obtained from the tail of this rat. With direct light and the high dry lens of the microscope a number of large trypanosomes were first noted struggling to free themselves from masses of blood platelets. Other trypanosomes were single and appeared normal. The preparation was then examined with dark-field illumination. Nothing noteworthy was observed. In the second preparation some agglutination of the parasites was observed. With dark-field illumination several large motionless trypanosomes were seen floating in the serum while others were rapidly being ingested by macrophages. Actual seizure of the immobilized trypanosome by a macrophage was not observed, but ingestion always began at the posterior end of the trypanosome, the flagellar end being the last to disappear within the phagocytic cell. Marked agglutination was found in the third preparation with continued phagocytosis of single parasites. A very few single parasites, uniform in size, were found in the fourth preparation but none was seen in the fifth and the several subsequent preparations. The parasites had been removed from the blood within about 15 minutes.

The same experimental rat was then similarly reinjected with the remaining 0.5 cc. of the inoculum. The first drop of blood again swarmed with single trypanosomes. The same phenomena in recovery were again observed. The rat died about 15 minutes after the injection was made at which time a few single trypanosomes were present in the blood.

Blood smears made before and immediately after the first inoculation show nothing of particular note. Markedly increased phagocytosis occurs in the later smears, but the cytoplasmic inclusions of the mononuclear leucocytes cannot be identified with certainty as remnants of trypanosomes. Trypanosomes occur frequently within clumps of blood platelets, an observation which would not be considered of importance or noteworthy had living trypanosomes not been seen struggling to free themselves from masses of platelets in the fresh preparations. Remnants of trypanosomes, ruptured cells and free undulating membranes with attached

kinetoplasts are fairly numerous in the second and third smears made. As in earlier experiments, these remnants are more or less evenly distributed over the slide, are unattached and the majority indicate that they are vestiges of dividing cells. As none of these vestiges was seen in the fresh preparations, particularly looked for here and elsewhere with dark-field illumination, it is believed that they are remnants of the large sensitized or killed trypanosomes seen floating in the fresh preparations which, because of the action of the trypanocidal antibody, have been rendered particularly fragile and have burst on the slide during the technical preparation of the blood smear. We obtained no evidence of extracellular lysis of the parasites.

DISCUSSION

From the experimental findings just described it is apparent that trypanosomes (*T. lewisi*) pass as readily through lymph nodes of immune rats as they do through lymph nodes of a normal, susceptible rat. When these parasites are injected intraperitoneally in an immune rat they suffer no apparent injury during a temporary wait in the peritoneal cavity or from passing through its lymph nodes.

Reinfection is usually of short duration, lasting, in our experiments, from a few minutes to several days, but usually not longer than 4 days. The life span of the parasites in the circulating blood of the immune rat is chiefly determined by two important factors: (1) the titer of the trypanocidal antibody and (2) the particular phase of the infection, *i. e.* age of the parasite, at the time of transfer. The results of our experiments indicate that dividing trypanosomes are particularly vulnerable to the trypanocidal antibody. They are almost immediately sensitized with it, being killed or at least immobilized, and are then engulfed by blood macrophages. Adult trypanosomes, however, appear more resistant to the trypanocidal antibody and are not immobilized or killed by it, but when the titer of the antibody reaches a certain point, the adult parasites are agglutinated and the living, agglutinated masses are then mechanically filtered out and disposed of in the internal organs. Dividing cells may also be agglutinated along with adult forms. While we have not been able to recognize vestiges of trypanosomes in tissue macrophages we have found remnants of agglutinated parasites in the small vessels of the liver (fig. 5). This evidence and the presence of an increased amount of debris in the Kupffer cells furnish convincing evidence that the agglutinated masses of trypanosomes are removed from the circulation chiefly in the liver, and prob-

ably also in the spleen, and that they are finally disposed of in these organs by the phagocytic action of the elements of the reticulo-endothelial system.

Thus, the mechanics in recovery from reinfection with *T. lewisi* are strikingly and fundamentally different from those described in recovery from the initial infection. According to Taliaferro,² and others, there are three manifestations of resistance against the parasite in the course of the first infection: (1) the retardation and final inhibition of reproduction of the trypanosomes by about the 10th day by a development of an acquired immunity involving an antibody now known as ablastin; (2) a sudden destruction of most of the circulating trypanosomes by the action of a trypanocidal antibody and (3) the total disappearance of the parasites which terminates the infection and occurs from a week to several months after the first drop in numbers. Although the specific action of the trypanocidal antibody has not been demonstrated in the whole blood of recovered rats, the current opinion seems to be that it functions as an extracellular lysis.

Our experiments have given no indication that ablastin functions in recovery from reinfection. It is true that the parasitic population in the blood of the immune rat does not increase, but this failure is not due to an inhibition of reproductive activity. On the contrary, reproduction may be initiated, or may continue for some time in the circulation. However, the dividing cells are particularly susceptible to the action of trypanocidal antibody and they succumb as quickly as they are formed. Thus, an increase in the parasite population is checked, but reproductive activity is not inhibited.

The results of the present experiments leave little doubt as to the precise functions of the trypanocidal antibody. Direct observations have shown that it sensitizes the dividing cells which are thus rendered vulnerable to the phagocytic cells of the blood, and that it agglutinates the adult parasites which are then mechanically removed from the circulation. It is possible that we are dealing with two distinct antibodies, (1) an opsonin which acts specifically on dividing trypanosomes and (2) an agglutinin which agglutinates both adult and dividing forms. At present, however, we prefer to regard the trypanocidal antibody as a single sensitizing agent, the different manifestations being due to differences in its titer and to differences in susceptibility of dividing and adult parasites.

The results we obtained from experimental rat 4 clearly illustrate how strictly the trypanocidal antibody is localized in the blood. On April 2 this rat had, from all appearances, just recovered from a

previous infection when it was again inoculated intraperitoneally with millions of trypanosomes. On the following day fluid aspirated from the peritoneal cavity swarmed with trypanosomes which produced infection when transferred to another rat. No trypanosomes were found at the time in the blood of rat 4, nor did the blood carry infection when it was inoculated into young rats. From the results of our earlier studies,^{7,8} it is obvious that trypanosomes were constantly arriving in the blood from the peritoneal cavity at the time. It would, therefore, appear that the titer of the antibody in this hyperimmune animal was particularly high and that the parasites were destroyed as soon as they were exposed to the antibody, *i. e.* as soon as they entered the blood stream.

SUMMARY

Experiments concerning factors in the defense mechanism against reinfection with *Trypanosoma lewisi* are reported. It has been shown that this parasite passes readily and uninjured through the lymph nodes of rats spontaneously recovered from a previous infection.

It is not apparent that ablastin plays any role in recovery in reinfection. The disposal of the trypanosomes depends exclusively on the action of a humoral trypanocidal antibody to which dividing parasites are highly susceptible and to which the adult forms are relatively resistant. Dividing cells appear in the circulating blood, but are soon killed or immobilized by the antibody and they are then destroyed in the circulating blood by phagocytosis. At a sufficiently high titer of the antibody the adult parasites are agglutinated, but not killed. The agglutinated masses of living parasites are mechani-

cally removed from the circulating blood and then destroyed in the liver and probably also in the spleen.

The possibility of two distinct trypanocidal antibodies functioning in recovery from reinfection is considered.

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PLATE I

FIGS. 1, 2, 3, and 4 stained by Wright's method. FIG. 5 stained with eosin methylene blue.

FIG. 1. Agglutination of two trypanosomes showing the characteristic manner of attachment. $\times 900$.

FIG. 2. Two large masses of agglutinated trypanosomes, the attachment slightly distorted by drying of the film. $\times 900$.

FIG. 3. A ruptured dividing trypanosome adjacent to an uninjured adult parasite. $\times 1160$.

FIG. 4. A vestige of a trypanosome showing a lobed kinetoplast and surrounded by uninjured adult parasites. $\times 1160$.

FIG. 5. Section of liver showing remnants of an agglutinated mass of trypanosomes in a blood vessel. Only one free flagellum is clearly seen in the photograph. $\times 1600$.







